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COMPLETE SPECIFICATION

Preparation of Partially Depolymerized Hyaluronic Acid and Therapeutic Compositions Thereof

We, AMERICAN HOME PRODUCTS CORPORATION, a Corporation organized and existing under the Laws of the State of Delaware, United States of America, of 22, East 40th Street, in the City, County, and State of New York, United States of America, (Assignee of Joseph Seifter and David Harry Baeder), do hereby declare the invention, for which we pray that a patent may be granted to us, and the method by which it is to be performed, to be particularly described in and by the following statement:—

This invention relates to partially depolymerized hyaluronic acid, which is useful as a spreading and lipemia-clearing agent.

In the injection of therapeutic agents, prompt spreading of the agents in the tissues is frequently desirable. This is particularly so in the case of local and regional anesthetics and in hypodermoclysis. Recently the enzyme hyaluronidase has been successfully used as a spreading agent both on animals and clinically on human beings. This action is usually attributed to the decrease in viscosity of the ground substance which results from depolymerization of the hyaluronate by the enzyme. The effect is to decrease the normal resistance of the barrier. Another possibility is that the enzyme releases partially depolymerized hyaluronic acid (referred to below as PDHA) which then acts as a transport agent, ion-exchanger, or a protective colloid and peptizing agent, aiding the dispersion of materials in the tissues.

In experiments to determine this point, we have discovered that partially depolymerized hyaluronic acid (PDHA) facilitates the spread and absorption of injected materials in animal and human tissues, that this effect is a function of the degree of depolymerization, and that it can be employed in drug injections in living tissues.

While hyaluronidase is an effective spreading agent and has been successfully used therapeutically, it has several disadvantages not shared by PDHA. Hyaluronidase is a protein and tends to be unstable in aqueous solution. As a protein it is capable of producing sensitivity reactions and of neutralizing antibodies in the blood, so there is a possibility that prolonged use in the same patient may result in the negation of its usual properties. PDHA is non-antigenic and more stable in solution.

According to one feature of the present invention the method of producing a material having the property of promoting the spread of therapeutic liquid introduced into living animal tissues comprises partially depolymerizing hyaluronic acid to a degree corresponding to that obtained by incubating a 5 percent sodium hyaluronate solution in physiological saline with 20 TRU hyaluronidase per milligram of hyaluronic acid at 38°C. for a period in the range of 5 to 60 minutes. This partial depolymerization of hyaluronic acid (designated as HA) can be effected by incubating it in the form of an alkali metal salt in aqueous solution with hyaluronidase under controlled time and temperature conditions, and then deactivating and removing the enzyme. The resulting solution of PDHA is mixed with the material to be injected and the mixture injected.

The invention accordingly includes the method of partially depolymerizing hyaluronic acid which comprises dissolving a hyaluronate of an alkali metal in physiological saline to form a 5 percent solution of the former, adding hyaluronidase in a ratio of 20 TRU per milligram of hyaluronate, incubating the mixture at 38°C. for a period in the range of from 5 to 60 minutes, heating the mixture at a temperature and for a time sufficient to deactivate the hyaluronidase, cooling, and separating the precipitated deactivated hyaluronidase from the solution of the desired partially depolymerized hyaluronic acid.

A suitable degree of depolymerization of 90

HA is reached by incubating, for example, 5 percent sodium hyaluronate in physiological salt solution with the hyaluronidase at 38°C. for 15 minutes, after which the enzyme is deactivated and removed by autoclaving and centrifuging. The supernatant liquid contains the desired PDHA.

Longer incubation reduces the spreading effect; for example, incubation for 60 minutes lessens it by at least 50 percent. The degree of depolymerization which will give a product useful for the purpose indicated above corresponds to that obtained by incubation under the described conditions for a period of at least 5 minutes and less than 60 minutes. The depolymerization may be effected otherwise than by hyaluronidase, as by non-enzymatic procedures involving, for example, treatment with ascorbic acid or with hydrogen peroxide and a metal, especially copper.

In addition to its spreading action, PDHA has been found to have a lipemia-clearing action when administered intravenously, subcutaneously or orally.

The invention broadly includes a solution in physiological saline substantially free of hyaluronidase activity of hyaluronic acid which has been partially depolymerized to a degree corresponding to that obtained by incubating a 5 per cent sodium hyaluronate solution in physiological saline with 20 TRU hyaluronidase per milligram of hyaluronic acid at 38°C. for a period in the range of 5 to 60 minutes.

The invention also includes the method of making a preparation of a drug for parenteral administration having improved ability for spread and absorption which comprises admixing with the drug a physiological salt solution of hyaluronic acid which has been partially depolymerized to the degree just specified above in the form of a non-toxic alkali metal salt in an amount sufficient to produce the desired spread and absorption.

Examples of the preparation and physiological effects of partially depolymerized hyaluronic acid according to the present invention are given below, but these are intended to be illustrative only and not to limit the invention.

EXAMPLE 1.

Preparation of PDHA

A 5% solution of sodium hyaluronate (HA) was prepared by dissolving 1,250 mg. of streptococcal HA in 25 ml. of physiological salt solution U.S.P. The mixture was placed in a water bath at 38° ± 1°C. and stirred until the hyaluronate was in solution. To the HA solution 25 mg. of hyaluronidase (1,000 TRU/mg.) was added and stirred until it dissolved. The HA-hyaluronidase mixture was kept in the water bath for 15 minutes, after which it was removed and placed in a steam pressure autoclave for 40 minutes (T=104°C.) At the end of 40 minutes the pressure in the autoclave was permitted to return to normal by shutting off the steam supply. The precipitated hyaluronidase was removed by centrifugation at 1,800 R.P.M. (10 cm. radius) for 15 minutes. The supernatant fluid, containing the partially depolymerized hyaluronic acid as its sodium salt (PDHA), was assayed by the turbidimetric assay for residual hyaluronidase. The assay showed less than 0.1 TRU hyaluronidase/ml of solution. This solution containing the depolymerized HA, has pronounced spreading action and facilitates absorption of solutions injected subcutaneously. If the depolymerization by the enzyme is prolonged to 60 minutes the spreading action is lessened by at least 50%.

The effect of duration of incubation on the spreading activity of PDHA is shown in the following table. The PDHA was prepared as above except that the incubation period was varied. Doses of 5 mg. in 0.1 ml. test solution and 0.1 ml. 1% trypan blue in physiological salt solution were injected intradermally into anesthetized rats, and the area of spread was determined after a lapse of 60 minutes as described in Example. 2 The results are given in the following table in comparison with control injections of physiological salt solution (PSS) and of 1,000 TRU of hyaluronidase. It will be seen that in this series the spreading effectiveness of PDHA increases with incubation time from 5 minutes to 20 minutes, falls to a plateau at 30–50 minutes, and starts decreasing again at 55 minutes. Effective spreading is obtained in the range from 5 minutes to not over 60 minutes, but maximum effectiveness is obtained in the preferred range 10–25 minutes.

TABLE I.

Relation of Incubation Time of Hyaluronic Acid with Hyaluronidase to Spreading Effectiveness of Partially Depolymerized Hyaluronic Acid (PDHA)

Composition Injected	*Area of Spread (sq. mm.) After 60 Min.*
Physiological Salt Solution (PSS)	255.0
Hyaluronidase, 1,000 TRU	596.2
PDHA incubated 5 minutes	462.6
" " 10 "	565.0
" " 15 "	981.4
" " 20 "	1,047.4
" " 25 "	883.3
" " 30 "	483.4
" " 35 "	447.2
" " 40 "	441.9
" " 45 "	433.5
" " 50 "	417.4
" " 55 "	399.9

EXAMPLE 2.

Comparison of the Spreading Action of PDHA with that of Hyaluronidase

Spreading tests were made on albino

rabbits of both sexes weighing between 2.3 kg. and 2.9 kg. The intradermal injections were made through a 26 guage, one-half inch needle into the lumbar and scapular regions clipped free from hair. The injections consisted of 0.1 ml. of 1% trypan blue in physiological salt solution (PSS) mixed with 0.1 ml. of test solution. Measurements of the large diameter of spread (D) and the small diameter of spread (d) were made with a vernier caliper immediately after injection and 15, 30, and 60 minutes later. The area of spread was computed from the formula for the area of an ellipse, A sq. mm. $= \frac{D \times d \times \pi}{4}$. Each concentration of a substance was tested on a minimum of 6 rabbits. Each rabbit received a control injection of PSS. The injection sites for the control and materials to be tested were randomized.

In the following table a comparison is made of the spreading effect of increasing doses of hyaluronidase and of 5 mg. streptococcal PDHA hydrolyzed 15 minutes and hydrolyzed 60 minutes.

TABLE II.

Spreading Effect of Hyaluronidase and Streptococcal PDHA in Rabbits Injected 1% Trypan Blue

Dosage	*Hyaluronidase—TRU* injected*						
	control (PSS)	100	1,000	2,000	5,000	10,000	20,000
Mean area of spread, mm.²	153.5	197.2	388.2	483.0	643.6	675.6	587.2

	PDHA injected—hydrolyzed for	
	15 min.	*60 min.*
Dosage	5 mg.	5 mg.
Mean area of spread, mm.²	533.1	241.3

*TRU=turbidity reducing units.

It is seen that 5 mg. 15-minute PDHA has a greater effect than 2,000 TRU and less than 5,000 TRU of hyaluronidase. The 60-minute PDHA had somewhat less than half this effect.

EXAMPLE 3.

Undepolymerized hyaluronic acid (HA) had practically no useful spreading effect, as compared with the control of Table II. The results of tests on rabbits with HA from different sources and in different amounts is shown in Table III.

TABLE III.

Spreading Effect of HA on Rabbits Injected with 1% Trypan Blue—mm.² in 60 Minutes

Amount injected, μg	*control*	50	100	1,000	2,000	5,000
PSS	153.5	—	—	—	—	—
Streptococcal HA	—	147.1	217.2	198.1	163.7	100.3
Umbilical cord HA	—	160.7	193.5	202.2	149.5	112.9
Vitreous humour HA	—	202.2	194.7	171.5	142.3	138.3

EXAMPLE 4.

Hypodermoclysis

The ability of PDHA to facilitate a hypodermoclysis of PSS in rabbits is shown in Table IV. It will be seen that 150 mg. of PDHA was as effective as 150 TRU of the enzyme. In other experiments 50 and 100 mg. of PDHA added to the clysis was without facilitating effect.

TABLE IV.

Effect of Hyaluronidase and PDHA on Hypodermoclysis in Rabbits

	Time for Hypodermoclysis—minutes
Saline	47
Hyaluronidase, 150 TRU	19
Saline	51
PDHA, 150 mg.	18

EXAMPLE 5.

Spread of an X-Ray Contrast Medium

Facilitation of spread and absorption of an X-ray contrast medium in guinea pigs is shown in Table V. It can be seen that 150 TRU of hyaluronidase or 150 mg. of PDHA increased the area of spread and the rate of absorption and appearance of the opaque material in the urinary bladder by twofold.

TABLE V.

*Effect of Hyaluronidase and PDHA on Absorption and Excretion of "Urokon"**

Treatment Group	*Spread mm.² at time of injection*	*Appearance in Urinary Bladder (Minutes)*
Saline + "Urokon"	Mean 4.4	30

Treatment Group	Spread mm.[a] at time of injection	Appearance in Urinary Bladder (Minutes)
5 150 TRU Hyaluronidase + " Urokon " + 10 150 mg. PDHA + " Urokon "	Mean 10.4 / Mean 11.4	16.3 / 16.9

[a]An X-ray contrast medium sold under the 15 Registered Trade Mark " Urokon."

EXAMPLE 6.

The effect of PDHA on an injected local anesthetic was tested on four human subjects. Threshold amounts of a proprietary 20 local anesthetic " Wycaine " (a substituted glycinamideethanolamine derivative) were injected into the subjects both with and without the addition of PDHA. Since only threshold amounts were used, it was impossible to 25 measure the spreading effect directly because increased spreading would result in subthreshold concentration at the periphery. However, the duration of anesthesia offered an indirect measure of spreading, absorption 30 being inversely proportional to the extent of spreading and rate of absorption. From the following table it will be seen that PDHA facilitates the spread and absorption of the injected anesthetic, the duration of anes- 35 thesia being substantially halved by its presence.

TABLE VI.

Duration in Minutes of Local Anesthesia in Human Subjects Injected with 1 ml. of 0.02% " Wycaine " with and without the Addition of 1 mg. PDHA

Subject	Without PDHA	With PDHA
1	140	70
2	> 125	55
3	> 125	70
4	100	70

EXAMPLE 7.

This example illustrates the lipemia-clearing effect of PDHA on dogs. Lipemia was established for the intravenous test in 3 male and 3 female fasted dogs by the intragastric administration of 20-25 ml./kg. of cottonseed oil. The extent of lipemia in the plasma was determined by determining its light transmission at 650 mμ in a Beckman quartz spectrophotometer using silica microcuvettes at 22°C. Lipemic plasma transmitted 35% or less of the incident light, while normal plasma transmitted 85% or more. The lipemia-clearing effect of intravenously adminstered PDHA is shown in Table VII.

In the table the first column indicates the dosage, the second column transmission of the plasma before treatment, the third column transmission 60 minutes after administration of cottonseed oil, and succeeding columns transmission after the number of minutes indicated in the headings. The PDHA was injected at the 60 minute interval.

TABLE VII.

Lipemia-Clearing Effect of Intravenous PDHA on Dogs: Percentage Light Transmission of Plasma (Average of 6 Dogs) at the Indicated Time in Minutes after Adminstration of Cotton Seed Oil—PDHA Injected at 60 Minutes

Dosage	Untreated	60	90	120	150	180	210	240	300	360 min.
20 mg./kg.	70	55	77	85		88		59	46	37
10 mg./kg.	94	55	92	92	84	64		52	41	8

The oral effect of PDHA is shown in Table VIII. In this case no cotton seed oil was administered, but the plasma taken from dosed dogs at hourly intervals was added to lipemic plasma in the ratio 0.1 ml. : 0.5 ml., the mixture held for 5 minutes at room temperature (22°C.) and light transmission determined as above.

TABLE VIII.

Lipemia-Clearing Effect of Oral PDHA on Dogs: Percentage of Light Transmission of Plasma at the Indicated Time in Minutes after Start

Dosage	Untreated	60	120	180	240	300 min.
50 mg./kg.	86	66	87	95	64	37

From the preceding disclosure it will be seen that we have discovered a new product useful in various ways in animal experimentation and in animal and human therapy, and a method of preparing it.

The terms hyaluronic acid (HA) and partially depolymerized hyaluronic acid (PDHA) are used in the specification and claims to include both the free acids and their alkali metal salts. They also include, respectively, hyaluronic acid and partially depolymerized hyaluronic acid regardless of the source of preparation, viz: umbilical cord, vitreous humour or bacteria.

What we claim is:—

1. The method of producing a material having the property of promoting the spread of therapautic liquids introduced into living animal tissues which comprises partially depolymerizing hyaluronic acid to a degree corresponding to that obtained by incubating a 5 percent sodium hyaluronate solution in physiological saline with 20 TRU hyaluronidase per milligram of hyaluronic acid at 38°C. for a period in the range of 5 to 60 minutes.

2. The method of partially depolymerizing hyaluronic acid which comprises dissolving a hyaluronate of an alkali metal in physiological saline to form a 5 percent solution of the former, adding hyaluronidase in a ratio of 20 TRU milligram of hyaluronate, incubating the mixture at 38°C. for a period in the range of 5 to 60 minutes, heating the mixture at a temperature and for a time sufficient to deactivate the hyaluronidase, cooling, and separating the precipitated deactivated hyaluronidase from the solution of the desired partially depolymerized hyaluronic acid.

3. The method defined in Claim 2 in which the incubation period is in the range 10-25 minutes.

4. A solution in physiological saline substantially free of hyaluronidase activity of hyaluronic acid which has been partially depolymerized to the degree specified in Claim 1.

5. A solution as defined in Claim 4 containing an injectable therapeutic agent.

6. A solution as defined in Claim 4 containing an injectable diagnostic agent.

7. A solution as defined in Claim 4 containing an injectable X-ray contrast agent.

8. A solution as defined in Claim 4 containing an injectable anesthetic agent.

9. The method of making a preparation of a drug for parenteral administration having improved ability for spread and absorption, which comprises admixing with the drug a physiological salt solution of hyaluronic acid which has been partially depolymerized to the degree specified in Claim 1 in the form of a non-toxic alkali metal salt in an amount sufficient to produce the desired enhancement of spread and absorption.

10. The methods of making partially depolymerized hyaluronic acid as herein particularly described and illustrated by the examples.

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ERRATA

SPECIFICATION NO. 769,287

Page 3, line 3, *for* "guage" *read* "gauge".

Page 4, line 9, *delete* "+".

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